UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

DATE: 11/6/2017

SUBJECT: **Metolachlor/S-metolachlor**: Report of the Cancer Assessment Review Committee (5th Evaluation)

PC Code: 108800 S-Metolachlor; 108801 Metolachlor
Decision No.: 518226
Petition No.: N/A
Risk Assessment Type: Cancer Assessment
TXR No.: 0057654
MRID No.: N/A

DP Barcode: N/A

Registration No.: N/A
Regulatory Action: PRIA
Case No.: N/A
CAS No.: 87392-12-9; 51218-45-2
40 CFR: N/A

FROM: Sarah Dobreniecki, Executive Secretary
Cancer Assessment Review Committee
Health Effects Division (7509P)

THROUGH: Gregory Akerman, Chair
Anwar Dunbar, Co-Chair
Cancer Assessment Review Committee
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TO: Jessica Kidwell, EPS
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The Cancer Assessment Review Committee met on July 19, 2017 to re-evaluate the cancer classification of metolachlor/S-metolachlor in accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005). The final Cancer Assessment is attached.

CANCER ASSESSMENT DOCUMENT

FIFTH EVALUATION OF THE CARCINOGENIC POTENTIAL OF METOLACHLOR (PC CODE 108801)/S-METOLACHLOR (PC CODE 108800)

11/6/2017

CANCER **A**SSESSMENT **R**EVIEW **C**OMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

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EXECUTIVE SUMMARY

The Cancer Assessment Review Committee met on July 19, 2017 to re-evaluate the cancer classification of metolachlor in accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005). In 1994, the Health Effects Division Carcinogenicity Peer Review Committee (CPRC) concluded that the classification of metolachlor should remain as Group C - possible human carcinogen - and recommended that a Margin of Exposure (MOE) methodology be used for the estimation of human risk, and not the Q_1* approach. This Group C classification was based on increased liver tumors seen in female rats following exposure to the racemic metolachlor. No treatment-related tumors were seen in male rats or male or female mice. Since that time, the registrant conducted new mechanistic studies to support a human relevance framework analysis for a mitogenic mode of action (MOA) for liver tumors in female rats involving the activation of the constitutive androstane receptor (CAR). The registrant has requested that EPA reassess the cancer classification for metolachlor based on the available MOA data. The registrant has submitted new studies containing MOA data on S-metolachlor, which the agency has concluded to be of comparable or less toxicity to metolachlor so that studies can be used interchangeably for toxicological decision for risk assessment (Dobozy, V., 2001a, Dobozy, V., 2001b).

The new studies were considered in the context of the registrant's proposed CAR-mediated mitogenic MOA for the liver tumors in female rats seen following long term exposure to metolachlor. The CARC concluded that the *in vitro* and *in vivo* data adequately demonstrated dose and temporal concordance to support key events for the MOA leading to liver tumors in female rats. In the absence of a long-term carcinogenicity study with S-metolachlor, the tumorigenic effects of metolachlor can be reasonably explained by CAR activity demonstrated in the MOA for S-metolachlor. This is supported by the comparable effects of S-metolachlor and metolachlor on CYP2B expression/BROD activity and liver hypertrophy.

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March 2005), the CARC concluded that metolachlor/ S-metolachlor should be reclassified as "Not Likely to be Carcinogenic to Humans" at doses that do not induce cellular proliferation in the liver. This classification was based on convincing evidence that a mitogenic mode of action for liver tumors in female rats has been established and that the carcinogenic effects have been demonstrated as a result of a MOA dependent on CAR activation. There is no concern for mutagenicity.

Based on this cancer classification, the quantification of cancer risk using a Q_1* approach is not required. A non-linear approach (i.e., RfD) would adequately account for all the chronic toxicity, including carcinogenicity, that could result from exposure to metolachlor/S-metolachlor. The RfD should be protective of the dose which induced hepatocellular proliferation (150 mg/kg/day or 3000 ppm) in the female rat.

I. INTRODUCTION

On July 19, 2017, the Cancer Assessment Review Committee (CARC) of the Health Effects Division (HED) of the Office of Pesticide Programs (OPP) met to re-evaluate the carcinogenic potential of metolachlor based on data submitted to support the registrant's proposed CAR mediated mitogenic mode of action for liver tumors in female rats.

II. BACKGROUND INFORMATION

Metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide] and S-metolachlor [S-2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide] are selective, chloroacetanilide herbicides that are applied to a variety of crops as a preplant, preplant-incorporated (PPI), pre-emergence, or post-emergence-directed application, primarily for the control of grass weeds. Metolachlor is registered to Sipcam Agro USA, Inc., Drexel Chemical Company, and TRI Chemical, Inc. (formerly Cedar Chemical). S-metolachlor is currently registered to Syngenta Crop Protection. Metolachlor is a racemic herbicide that consists of 50% each of the R-enantiomer and the S-enantiomer, which is the herbicidally active isomer. S-metolachlor is an isomer enriched form of metolachlor, comprised of 88% S-isomer and 12% R-isomer.

The existing toxicological database is comprised primarily of studies conducted with metolachlor. The toxicology database for S-metolachlor consists of bridging data, which include an acute toxicity battery, subchronic toxicity studies in rats and dogs, developmental toxicity studies in rats and rabbits, a mutagenicity battery, metabolism studies and an immunotoxicity study. Based on a comparison of findings in the two databases by HED's Hazard Identification Assessment Review Committee (HIARC) as well as the Metabolism Assessment Review Committee (MARC) (Dobozy, V, 2001a, Dobozy, V, 2001b), it was concluded that S-metolachlor is of comparable or less toxicity in comparison to the racemic mixture, metolachlor, and that studies with both chemicals can be used interchangeably for toxicology endpoint selection for risk assessment, including cancer classification and quantification of cancer risk . The combined metolachlor and S-metolachlor toxicity data bases are adequate to characterize the toxicity of both metolachlor and S-metolachlor for risk assessment purposes, with the exception of a required subchronic inhalation study. Risk assessments for either metolachlor or S-metolachlor consider uses, exposure, and toxicity of both chemicals.

Previously, the HED CPRC met on July 27, 1994 to discuss and re-evaluate the weight-of-the-evidence on metolachlor, with particular reference to its carcinogenic potential, based on additional data provided by the registrant (Dapson and Rinde, 1994, TXR No. 0011347). This was the fourth meeting to discuss the carcinogenic potential of metolachlor. These data were requested by the Agency in the third CPRC meeting (Dapson and Rinde, 1993, TXR No. 0010490). The classification of metolachlor at that time was Group C, with a recommendation that a low dose extrapolation model be applied to the animal data for the quantification of human

risk (Q_1*). The new data provided by the registrant consisted of metabolism and genotoxicity studies. The Peer Review Committee considered the criteria in the EPA's "Guidelines for Carcinogen Risk Assessment" [FR51: 33992-34003, 1986) for classifying the weight of evidence for carcinogenicity. Based on these data and in consideration of the full weight-of-the-evidence, the 4th CPRC concluded that the classification of metolachlor should remain as Group C - possible human carcinogen - and recommended that a margin of exposure (MOE) methodology be used for the estimation of human risk, and not the Q1* approach. The Group C classification was based on increased liver tumors seen in female rats following exposure to the racemic metolachlor. No treatment-related tumors were seen in male rats or male or female mice. Quantification of risk currently uses a non-linear approach (i.e. reference dose) to account for all chronic toxicity, including carcinogenicity.

Currently, the registrant has requested that EPA reassess the cancer classification for S-metolachlor/metolachlor. The registrant has submitted data with S-metolachlor to support a human relevance framework analysis for a mitogenic mode of action of liver tumors in female rats involving CAR activation.

On July 19, 2017, the CARC reconvened to evaluate these data submissions, which included the following proposed MOA framework and mechanistic studies:

> Scollon. E.; Green. R. (2016) S-Metolachlor - Human Relevance Framework Assessment of liver Tumor Induction in Female Rats. Project Number: TK0180349. Unpublished study prepared by Syngenta Crop Protection. LLC. 36p. MRID 49927701
>
> Elcombe. B. (2014a) Final Report: S-Metolachlor - Enzyme and DNA Synthesis Induction in Cultured Female Rat Hepatocytes. Project Number: TK0210728, CXR1334. Unpublished study prepared by CXR Biosciences. 25p. MRID 49927704
>
> Elcombe, B. (2014b) Final Report Amendment 1: S-Metolachlor - Enzyme and DNA Synthesis Induction in Cultured Female Human Hepatocytes. Project Number: TK0210728. CXR 1336. Unpublished study prepared by CXR Biosciences. 31p. MRID 49927702
>
> Elcombe, B. (2014c) Final Report: S-Metolachlor - Concentration Range Finding Study in Cultured Female Sprague Dawley Rat Hepatocytes. Project Number: TK0210728, CXR1333. Unpublished study prepared by CXR Biosciences. 15p. MRID 49927705
>
> Elcombe, B. (2014d) Final Report: S-Metolachlor - Concentration Range Finding Study in Cultured Female Human Hepatocytes. Project Number: TK0210728, CXR1335. Unpublished study prepared by CXR Biosciences. 20p. MRID 49927703
>
> Dhinsa, N. (2014) Final Report: S-Metolachlor - Oral (Dietary) Mechanistic Study to Evaluate Effects on the liver in the Female Rat. Project Number: TK0210720, BFI0194.

Unpublished study prepared by Sequani limited. 566p. MRID 49927706

Omiecinski, C. (2014) Final Report: S-Metolachlor - CAR3 Transactivation Assay with Mouse, Rat and Human CAR. Project Number: TK0219524. Unpublished study prepared by Pennsylvania State University. 26p. MRID 49927707

These new studies were considered when re-evaluating the data to support proposed MOA for the liver tumors in female rats.

III. EVALUATION OF LIVER TUMORS AND MECHANISTIC STUDIES

In this document, HED previous review of the liver (female rats) tumor data (Dapson and Rinde, 1994, TXR 0011347) will be briefly discussed followed by the registrant's proposed mode of action for this tumor type.

A. Liver Tumors in Female Rats

The CARC previously determined the liver tumors at the high dose in female rats (3000 ppm) to be treatment-related.

The following information was extracted from the third CPRC report (Dapson and Rinde, 1993, TXR No. 0010490).

1. Combined Chronic Toxicity/Carcinogenicity Study with Metolachlor in CD [CD-Crl:CD(SD)BR] Rats

References:

Tisdel, M. (1983) Two-year chronic oral toxicity and oncogenicity study with metolachlor in albino rats. Hazleton Raltech, Inc., Madison, WI. Laboratory Study No.: 80030, May 2, 1983. MRID 00129377. Unpublished.

Hardisty, J.F. (1984) Two-year chronic oral toxicity and oncogenicity study with metolachlor in albino rats, Study No.: 80030; evaluation of liver tissues from male and female rats. Experimental Pathology Laboratories, Inc., Research Triangle Park, NC. Pathology summary, July 20, 1984. No MRID (follow up study). Unpublished.

Experimental Design: In a combined chronic toxicity/carcinogenicity study (MRID 00129377), groups of 60 CD [CD-Crl:CD(SD)BR] rats/sex/dose were administered metolachlor (95.3% a.i.; batch # FL-800362) in the diet at dose levels of 0, 30, 300, or 3000 ppm (equivalent to 0, 1.5, 15, and 150 mg/kg bw/day) for up to 24 months. Additional groups of 10 rats/sex/dose were administered metolachlor in the diet at 0 or 3000 mg/kg for 12 months; 5 rats/sex/dose were euthanized immediately after cessation of treatment, and the remaining 5 rats/sex/dose were

allowed to recover for four weeks.

Discussion of Tumor Data

For female rats, both the Hazleton and Hardisty pathology reports indicated significant increasing trends in liver neoplastic nodules (adenomas) ($p < 0.01$ for both reports) and combined liver adenomas and/or carcinomas ($p < 0.01$ for both reports). There were significant differences in the pair-wise comparisons of the controls with the 3000 ppm dose group for liver adenomas ($p < 0.05$) for the Hardisty report and for combined liver adenomas and/or carcinomas for both the Hazleton ($p < 0.05$) and Hardisty ($p < 0.01$) reports.

The statistical analysis of liver tumor rates was based upon the Exact trend test and the Fisher's Exact test for pair-wise comparisons since there were small occurrences of tumors and no significant statistical evidence of mortality with increasing doses of metolachlor.

The EPL internal peer review of the rat liver slides presented only those slides of animals on which Dr. Hardisty found liver lesions in 1984, in addition to six female control blanks. Given that this was not a complete evaluation of all of the liver slides of the Hazleton study, it would be inappropriate at this time to perform any statistical analyses on the results of the internal peer review.

Both the Hazleton and Hardisty pathology reports indicated significant increasing trends in liver neoplastic nodules (adenomas) ($p < 0.01$ for Hazleton; $p < 0.05$ for Hardisty) and combined liver adenomas and/or carcinomas ($p < 0.05$ for both reports) for male rats. There were no significant differences in the pair-wise comparisons of the controls with the dosed groups (Brunsman, L, 3/30/1993, HED Doc. No. 012793). These tumors were not considered to be treatment-related.

Table 1. Metolachlor - Charles River Crl:CD(SD)BR Rat Study

Female Liver Tumor Rates[+] and Exact Trend Test
and Fisher's Exact Test Results (p values)

Pathology Report by: Hazleton Raltech, Incorporated, May 2, 1983

Dose (ppm)

	0	**30**	**300**	**3000**
Neoplastic Nodule (Adenomas) (%)	0/58 (0)	0/60 (0)	1[a]/58 (2)	4/57 (7)
p =	0.005**	1.000	0.500	0.057
Hepatocellular Carcinomas (%)	0/58 (0)	0/60 (0)	0/58 (0)	2[b]/57 (4)
p =	0.059	1.000	1.000	0.244
Combined (%)	0/58 (0)	0/60 (0)	1/58 (2)	6/57 (11)
p =	0.000**	1.000	0.500	0.013*

+Number of tumor bearing animals/Number of animals examined, excluding those that died or were sacrificed before week 53.

[a]First neoplastic nodule (adenoma) observed at week 104, dose 300 ppm.
[b]First hepatocellular carcinoma observed at week 90, dose 3000 ppm.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

Table 2. Metolachlor - Charles River Crl:CD(SD)BR Rat Study

Female Liver Tumor Rates[+] and Exact Trend Test
and Fisher's Exact Test Results (p values)

Pathology Report By:
Dr. Jerry F. Hardisty
July 20, 1984

Dose (ppm)

	0	**30**	**300**	**3000**
Hepatocellular Adenomas (%)	0/58 (0)	1/60 (2)	2/58 (3)	6/57 (11)
p =	0.002**	0.509	0.248	0.0013*
Hepatocellular Carcinomas (%)	0/58 (0)	0/60 (0)	0/58 (0)	1/57 (2)
p =	0.245	1.000	1.000	0.496
Combined (%)	0/58 (0)	1/60 (2)	2/58 (3)	7/57 (12)
p =	0.001**	0.509	0.248	0.006**

+Number of tumor bearing animals/Number of animals examined, excluding those that died or were sacrificed before week 53.

Note: Significance of trend denoted at control.
Significance of pair-wise comparison with control denoted at dose level.
If *, then $p < 0.05$. If **, then $p < 0.01$.

B. Proposed Mode of Action for Liver Tumors

The registrant has proposed a mode of action for S-metolachlor-induced liver tumors in female rats (MRID 49927701) using the mode of action framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI).

The registrant has proposed that the available data for *S*-metolachlor support a MOA for liver tumors in female rats involving the following **key events**:

- Activation of the constitutive androstane receptor (CAR). **(Key Event #1)**
- An early, transient, increase in hepatocellular proliferation. **(Key Event #2)**
- Increased hepatocellular foci as a result of clonal expansion of spontaneously mutated (initiated) cells. **(Key Event #3)**
- Eventual progression to form liver tumors. **(Key Event #4)**

And the following **associative events**:

- Increased expression of genes encoding cytochrome P450s (CYPs), particularly CYP2B/3A isoforms. **(Associative Event #1)**
- Increases in smooth endoplasmic reticulum (SER) proliferation/hepatocellular hypertrophy. **(Associative Event #2)**
- Increased liver weight. **(Associative Event #3)**

The registrant proposes that at a sufficiently high free concentration of S-metolachlor in the liver, a cascade of events is initiated beginning with activation of the constitutive androstane receptor (CAR), resulting in increased expression of its target genes. CAR-regulated genes in rodents include a variety of genes that control DNA synthesis and the cell cycle. In the short term (up to 3 days of dietary administration), activation of CAR results in a transient increase in DNA synthesis and hepatocyte proliferation. Chronic maintenance of this CAR-activated state creates both an increased number of target cells for spontaneous mutation (initiation) to occur in and a permissive milieu in which spontaneously initiated cells can gain a selective growth advantage, which consequently leads to increased foci of cellular alteration and liver tumors. Activated CAR also induces the expression of a variety of xenobiotic metabolism genes, particularly those coding for cytochrome P450 (CYP) 2B and 3A isoforms, which results in SER proliferation leading to hepatocyte hypertrophy and, combined with the mitogenesis, to hepatomegaly. This mode of action hypothesis is described diagrammatically in Figure 1.

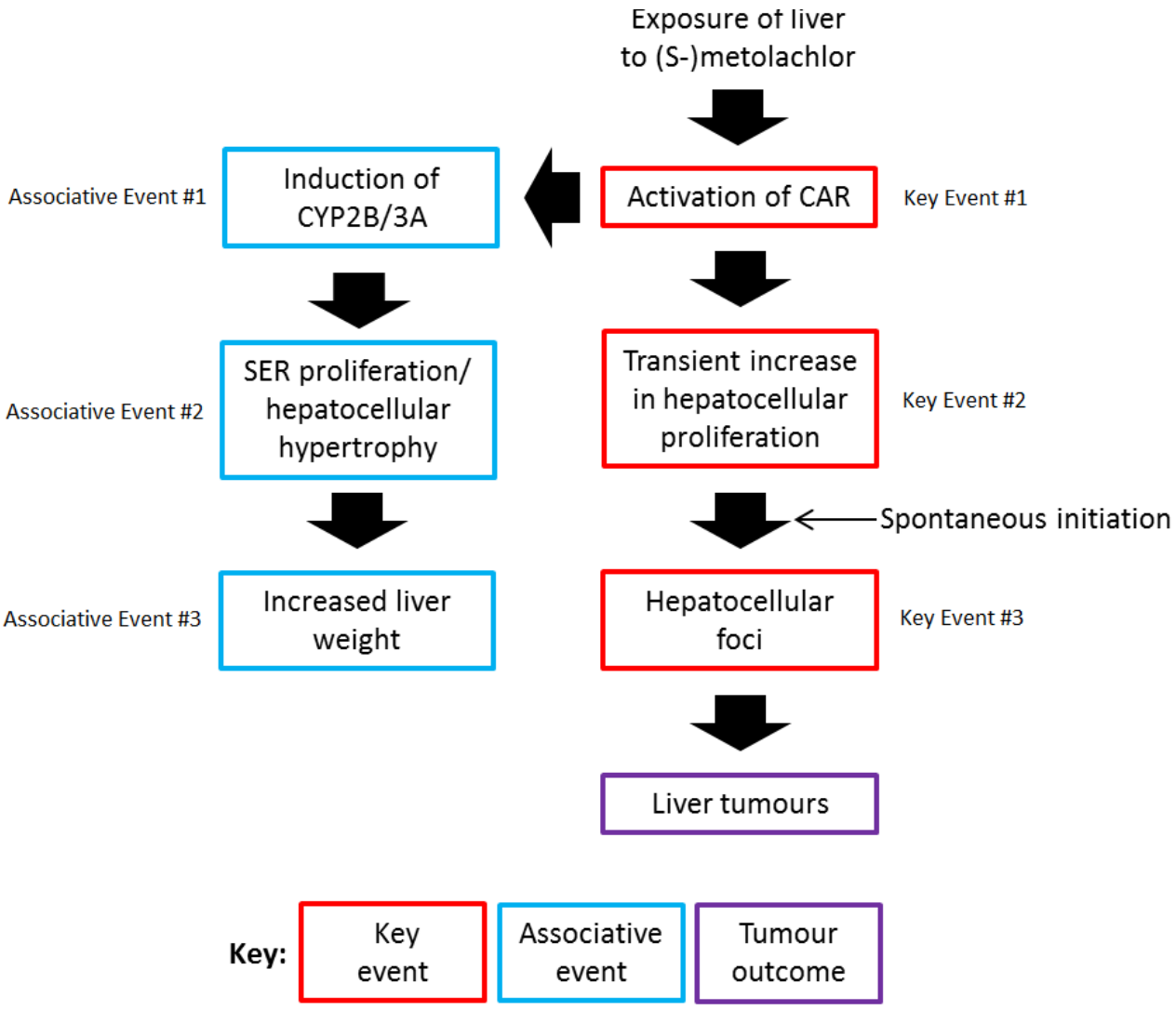

Figure 1. Mode of action hypothesis for metolachlor-induced liver tumors in female rats.

(modified from Figure 1 presented in MRID 49927701).

Mechanistic Studies submitted to support the proposed MOA

Key Event 1: Activation of the Constitutive Androstane Receptor (CAR)

In a non-guideline, *in vitro* study (Omiecinski, 2014, MRID 49927707), S-metolachlor was tested for its ability to activate the constitutive androstane receptor (CAR), a xenobiotic-sensing nuclear receptor that has been found to be involved in the development of mouse liver tumors induced by exposure to phenobarbital (PB) and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP). A luciferase reporter assay using COS-1 cells transfected with mouse, rat, or human CAR3 cDNA expression vectors, required cofactors, and a CYP2B6 response element-luciferase reporter construct was developed (Omiecinski- *et al.*, 2011) and used for this study. The cells were exposed to S-metolachlor at concentrations of 1, 3, 10, or 30 µM; the previously-tested, positive control, direct CAR activator, artemisinin, was used at the same concentrations; and model direct-acting substrates for mouse, rat, and human CAR were incubated at appropriate single concentrations. All activations were compared to the solvent control DMSO (<0.01% v/v). Light emissions from the luciferase reporter were quantified to measure the level of CAR3 activation.

The viability assays showed the chemicals did not cause cytotoxicity at the concentrations tested, with all viabilities ≥85%.

S-Metolachlor activated the CAR3 constructs in a dose-dependent manner. Human CAR3 was activated by 2.8- to 8.7-fold at concentrations ≥3 µM; mouse CAR3 was activated by 8.5- to 26.9-fold at concentrations ≥1µM; and rat CAR3 was activated by 51.3- to 57.1-fold at concentrations ≥10µM. The previously-tested, positive control compound, artemisinin, activated human CAR3 by 8.2-fold at 30 µM, mouse CAR3 by 3.9- to 12.9-fold at ≥3 µM, and rat CAR3 by 37.9- to 52.4-fold at ≥10 µM. The model positive control activators CITCO, TCPOBOP, and clotrimazole activated human, mouse, and rat CAR3 by 10.3-fold, 45.3-fold, and 95.4-fold, respectively. The indirect activator phenobarbital at 1 mM did not activate human CAR3, and activated mouse and rat CAR3 by 2.1-fold and 5.6-fold, respectively (**Figure 2**).

The CARC concluded that these data demonstrate that S-metolachlor directly activates rat, mouse and human CAR-mediated expression. (Key Event #1). Under the conditions of the assay, the activation of rodent CAR appeared to be stronger than human CAR.

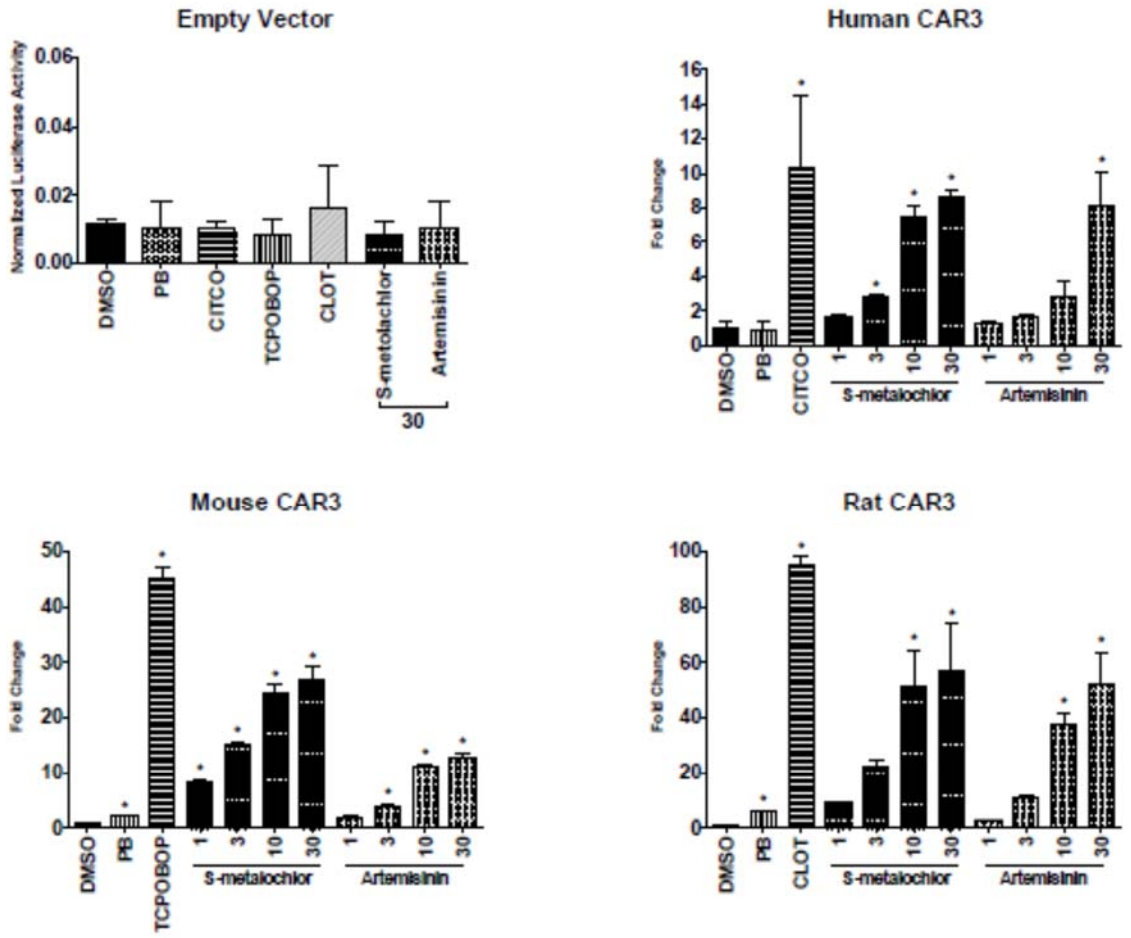

Figure 2: Results of *in vitro* rat, mouse and human CAR activation assays with *S*-metolachlor. *: Statistically significantly different from control with p≤0.01. Data represent means ± SD (n=4) (Omiecinski, 2014, MRID 49927707).

Key Event #2: Increase in hepatocellular proliferation.

Assessment of hepatocellular proliferation using 5-bromo-2-deoxyuridine (BrdU) incorporation technique. The following data support Key Event #2:

a. 14-day mechanistic study in female rats (MRID 49927706) – *S-metolachlor*

In a 14-day oral mechanistic study (Dhinsa, 2014, MRID 49927706), S-metolachlor (98.8% a.i-.; Batch # CAB2H12058) was administered to 50 Sprague Dawley [Crl:CD(SD)] female rats/dose in the diet at dose levels of 0, 300, 3000, or 5000 ppm (equivalent to 0, 32.2, 303.1, and 536.4 mg/kg/day) for up to 14 days. An additional group of 20 female Sprague Dawley rats was administered sodium phenobarbital (PB) in the diet at 1200 ppm (equivalent to 122.4 mg/kg/day) for up to 8 days as positive controls. Ten rats/sex/dose were euthanized on Days 2, 3, 4, 8, and 15. Hepatocellular proliferation was measured by incorporation of 5-bromo-2-deoxyuridine (BrdU). An increase in the BrdU labeling index (# BrdU labeled cells/1000 cells) was observed at 3000 ppm on Days 2, 3, and 4 and at 5000 ppm on Days 2 and 4 (Table 4). The BrdU labeling index was similar to control levels on Days 8 and 15. Treatment with 1200 ppm PB as a positive control increased the BrdU labeling index on Day 4; the labeling index was increased without significance on Day 8.

TABLE 4. Mean (±SD) BrdU cell counts in female rats treated with S-metolachlor in the diet for up to 15 days[a]						
Parameter		Dose (ppm)				
		0	300	3000	5000	Positive control[b]
Day 2						
Cell count	BrdU	9.0±5.0	11.4±5.3	18.6±9.8	19.0±13.5	---
	Normal	1039.2±22.9	1057.8±34.2	1073.2±50.0	1039.0±54.4	---
	Total	1048.2±23.7	1069.2±34.8	1091.8±48.0	1058.0±55.6	---
	BrdU/1000	8.566±4.762	10.644±4.890	17.108±9.122*	17.874±12.307*	---
Day 3						
Cell count	BrdU	6.3±3.5	8.0±4.4	25.5±11.3	9.7±9.8	---
	Normal	1043.1±31.6	1040.3±32.3	1025.8±26.9	1049.8±42.9	---
	Total	1049.4±30.8	1048.3±29.8	1051.3±26.7	1059.5±41.9	---
	BrdU/1000	6.022±3.401	7.692±4.279	24.218±10.665**	9.155±9.211	---
Day 4						
Cell count	BrdU	2.5±1.2	3.4±2.3	12.1±4.5	10.3±6.2	16.9±6.4
	Normal	1059.6±39.9	1052.0±42.0	1057.6±37.0	1046.7±46.0	1040.0±41.2
	Total	1062.1±40.2	1055.4±40.9	1069.7±34.4	1057.0±46.9	1056.9±41.3
	BrdU/1000	2.346±1.050	3.261±2.298	11.388±4.437**	9.715±5.906**	15.996±6.190**
Day 8						
Cell count	BrdU	5.2±3.1	5.9±3.6	5.5±3.5	4.5±4.2	10.5±7.9
	Normal	1049.6±41.6	1032.0±39.0	1041.6±43.9	1050.3±40.9	1062.0±50.5
	Total	1054.8±42.0	1037.9±38.7	1047.1±43.1	1054.8±40.7	1072.5±47.6
	BrdU/1000	4.921±2.866	5.697±3.506	5.286±3.448	4.274±4.109	9.894±7.525
Day 15						
Cell count	BrdU	3.7±2.1	3.6±1.7	3.6±4.0	4.0±2.7	---
	Normal	1068.8±39.6	1063.5±38.9	1064.1±48.9	1053.5±29.8	---
	Total	1072.5±40.4	1067.1±39.4	1067.7±48.2	1057.5±30.8	---
	BrdU/1000	3.428±1.856	3.360±1.583	3.399±3.659	3.757±2.465	---

a Data were obtained from Tables 1.1 through 1.5 and 2.1 through 2.2 on pages 475-479 and 481-482 in Appendix 10 of MRID 49927706.
b Positive control = 1200 ppm phenobarbital (PB). n=20 for Days 1-4; n=10 for Day 8.
* Significantly different from controls; $p<0.05$.
** Significantly different from controls; $p<0.01$.
--- Not recorded (all rats euthanized by Day 8)

b. 28-day study in rats (MRIDs 50396001 and 50396002) – *S-metolachlor and Metolachlor*

Groups of 5 rats/sex/dose (Tif: RAIf [SPF]) were administered metolachlor or S-metolachlor via the diet at concentrations of 0, 30, 300, 3000 and 5000 ppm for 28 days (Fankhauser, 1995, MRID 50396001; Persohn, 1995, MRID 50396002). An additional 3 rats/sex/dose were terminated after 7 days of dietary administration and an additional 5 rats/sex/dose (3000 and 5000 ppm only) were terminated after a 28-day recovery period. Only data for female rats are described below.

Hepatocellular proliferation was assessed in rats that received diet for 7 or 28 days by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). All S-metolachlor-receiving dose groups were investigated, as well as animals receiving 5000 ppm metolachlor.

No increases in hepatocellular proliferation were noted in any dose group.

c. 60-day mechanistic study in female rats (MRID 50396003) - *Metolachlor*

Groups of 15 female rats/dose/time point (Crl:CD [Sprague Dawley]) were administered metolachlor at dietary inclusion levels of 0 or 3000 ppm for 3, 5, 7, 14 or 60 days (Mainwaring, 2006, MRID 50396003). Hepatocellular proliferation, measured using a BrdU incorporation technique, and apoptosis, measured using terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL), were assessed in animals at each time point. Hepatic CYP activities/protein levels were measured in animals treated for 14 and 60 days only.

No consistent effects on either hepatocellular proliferation or apoptosis were noted in metolachlor-treated animals.

d. Other evidence of hepatocellular proliferation - *S-metolachlor and Metolachlor*

In their weight of evidence document (MRID 49927701), the registrant also presented evidence of hepatocellular proliferation measured as effects on S-phase replicative DNA synthesis in female rats following a single oral (gavage) dose of metolachlor or S-metolachlor. The registrant claimed these studies collectively demonstrated that oral administration of S-metolachlor to female rats can cause an increase in hepatocellular proliferation after short term dosing (Cifone, 1988, MRID 42043301; Ham, 1994a, MRID 43244003; Hertner 1995b, MRID 43928928). However, it should be noted that increased proliferation occurred at doses >300 - 1500 mg/kg, which are higher than the tumorigenic dose and not informative for dose concordance for the key event 2.

The CARC concluded that there is evidence of S-metolachlor induced hepatocellular proliferation (increased BrdU labelling) at ≥ 3000 ppm in female rats in the 14-day mechanistic study* (MRID 49927706) *(Key Event #2). Furthermore, this study showed a robust initial proliferative burst that subsided by Day 8, consistent with a CAR-mediated response.

Associative Event #1/ Associative Event #2:

Associative Event #1: Assessments of hepatic pentoxyresorufin-O-dependentylase (PROD) and 7-benzyloxyresorufin-O-debenzylase (BROD) activities. These reactions are catalyzed primarily by CYP2B/3A isoforms

and

Associative Event #2: Assessment of hepatic microsomal protein (increases in microsomal protein are indicative of SER proliferation)

a.* 14-day mechanistic study in female rats (MRID 49927706) – *S-Metolachlor

Liver biochemical data are presented in Table 5. Hepatic microsomal protein levels were increased ($p<0.05$) by 13% at 3000 ppm on Day 15, and by 14-25% at 5000 ppm on Days 4, 8, and 15. PROD and BROD activities generally increased with dose. PROD activity was increased ($p<0.01$) as follows: 44-59% at 300 ppm on Days 3 and 4; 404-1159% at 3000 ppm on

Days 2, 3, 4, 8, and 15; and 679-1629% at 5000 ppm on Days 2, 3, 4, 8, and 15. BROD activity was increased as follows: 95-161% at 300 ppm on Days 2, 3, and 4; 457-2618% at 3000 ppm on Days 2, 3, 4, 8, and 15; and 992-2618% at 5000 ppm on Days 2, 3, 4, 8, and 15. Treatment with 1200 ppm PB increased ($p<0.01$) hepatic microsomal protein levels by 19-29%, PROD activity by 2744-3488%, and BROD activity by 6961-7743%, on Days 4 and 8.

TABLE 5. Mean (±SD) PROD and BROD activities in female rats treated with S-metolachlor in the diet for up to 15 days.[a]					
Parameter	**Dose (ppm)**				
	0	**300**	**3000**	**5000**	**Positive control[b]**
Day 2					
Microsomal protein (mg/g liver)	24.3±2.23	29.8±3.98** (↑23)	27.6±4.67	28.1±4.04	---
PROD activity (pmol/min/mg protein)	19±3.9	25±6.4	111±37.5** (↑484)	148±88.1** (↑679)	---
BROD activity (pmol/min/mg protein)	38±9.2	74±25.6** (↑95)	573±274.1** (↑1408)	674±436.5** (↑1674)	---
Day 3					
Microsomal protein (mg/g liver)	26.0±2.34	26.4±2.83	27.1±2.38	26.7±3.68	---
PROD activity (pmol/min/mg protein)	17±1.7	27±6.9** (↑59)	214±74.6** (↑1159)	294±90.8** (↑1629)	---
BROD activity (pmol/min/mg protein)	33±7.8	86±19.0** (↑161)	897±346.1** (↑2618)	897±390.6** (↑2618)	---
Day 4					
Microsomal protein (mg/g liver)	27.4±3.88	28.3±1.83	30.1±1.70	31.1±2.19** (↑14)	32.7±2.72** (↑19)
PROD activity (pmol/min/mg protein)	16±2.8	23±5.3** (↑44)	132±32.3** (↑725)	186±53.2** (↑1063)	455±71.5** (↑2744)
BROD activity (pmol/min/mg protein)	28±7.0	63±16.7** (↑125)	350±113.9** (↑1150)	552±300.5** (↑1871)	1977±681.0** (↑6961)
Day 8					
Microsomal protein (mg/g liver)	24.7±2.22	24.5±2.07	27.5±2.49	30.9±4.41** (↑25)	31.9±1.96** (↑29)
PROD activity (pmol/min/mg protein)	26±3.6	23±3.7	169±56.0** (↑550)	270±103.4** (↑938)	933±329.3** (↑3488)
BROD activity (pmol/min/mg protein)	35±4.1	60±9.3	526±241.9** (↑1403)	533±319.3** (↑1423)	2745±1320.7** (↑7743)
Day 15					
Microsomal protein (mg/g liver)	24.2±2.11	24.1±1.96	27.4±2.76* (↑13)	27.9±2.62** (↑15)	---
PROD activity (pmol/min/mg protein)	23±4.2	30±5.4	116±29.7** (↑404)	188±59.6** (↑717)	---
BROD activity (pmol/min/mg protein)	65±18.6	87±21.2	362±125.9** (↑457)	710±157.9** (↑992)	---

a Data were obtained from Tables 1-5 on pages 448-452 in Appendix 9 of MRID 49927706. Percent difference from control (presented in parentheses) were calculated by the Investigators.

b Positive control = 1200 ppm phenobarbital (PB). n=20 for Days 1-4; n=10 for Day 8.

* Significantly different from controls; $p<0.05$.

** Significantly different from controls; $p<0.01$.

--- Not recorded (all rats euthanized by Day 8)

b. 28-day study in rats (MRIDs 50396001 and 50396002) – *S-metolachlor and Metolachlor*

Groups of 5 rats/sex/dose (Tif: RAIf [SPF]) were administered metolachlor or S-metolachlor via the diet at concentrations of 0, 30, 300, 3000 and 5000 ppm for 28 days (Fankhauser, 1995, MRID 50396001; Persohn, 1995, MRID 50396002). Livers from control and 5000 ppm group rats treated for 28 days were assessed for SER proliferation by electron microscopy. Moderate proliferation of the SER was noted in the treated females compared to the controls.

Treatment of female rats with either compound for 28 days at doses ≥3000 ppm resulted in marked increases in hepatic PROD activity and small increases in hepatic ethoxyresorufin-O-

deethylase (EROD) activity (this reaction is primarily catalyzed by CYP1A isoforms, but also by CYP2B and 3A isoforms [Burke et al., 1994]). These data are summarized in Table 6. In addition, treatment with either compound did not result in increased hepatic peroxisomal fatty acid β-oxidation. These data are consistent with the results of western immunoblots demonstrating that in females receiving either compound at ≥3000 ppm, there was induction of CYP3A1, but not CYP4A1/3 protein levels.

Table 6. Hepatic PROD and EROD activities in female rats following 28-day dietary administration of metolachlor or S-metolachlor.

Compound	Dose level (ppm)	Mean PROD activity (% of control)	Mean EROD activity (% of control)
Metolachlor	3000	2443***	234**
	5000	4471***	220*
S-metolachlor	30	57	105
	300	157	122
	3000	3071***	225**
	5000	6200***	248**

*, ** and ***: Statistically significantly different from control with p≤0.05, p≤0.01 and p≤0.001, respectively. PROD and EROD activities not determined for animals receiving 30 or 300 ppm metolachlor.

c. 60-day mechanistic study in female rats (MRID 50396003) - *Metolachlor*

Groups of 15 female rats/dose/time point (Crl:CD [Sprague Dawley]) were administered metolachlor at dietary inclusion levels of 0 or 3000 ppm for 3, 5, 7, 14 or 60 days (Mainwaring, 2006, MRID 50396003). Hepatic CYP activities/protein levels were measured in animals treated for 14 and 60 days only. Treatment of female rats for 14 or 60 days resulted in marked increases in hepatic PROD and BROD activities. Only minor increases in hepatic EROD and methoxyresorufin-O-demethylase (MROD) activities (reactions primarily catalyzed by CYP1A isoforms) were noted. These data are summarized in Table 7. There were no effects of treatments on peroxisomal fatty acid β-oxidation or lauric acid 11-/12-hydroxylation activities. These data are consistent with the results of Western immunoblots demonstrating marked inductions of CYP2B and CYP3A, no induction of CYP1A1 and only a very small increase in CYP1A2. These data are summarized in Table 8.

Table 7. Hepatic PROD, BROD, EROD and MROD activities in female rats following 14- or 60-day dietary administration of 3000 ppm metolachlor.

	14 Days	60 Days
Mean PROD activity (% of control)	902***	1590***
Mean BROD activity (% of control)	1336***	1918***
Mean EROD activity (% of control)	100	193*
Mean MROD activity (% of control)	114	162*

* and ***: Statistically significantly different from control with $p \leq 0.05$ and $p \leq 0.001$, respectively.

Table 8. Hepatic CYP isoform protein levels, measured by Western immunoblotting in female rats following 14- or 60-day dietary administration of metolachlor.

	14 Days		60 Days	
	0 ppm	**3000 ppm**	**0 ppm**	**3000 ppm**
CYP1A1	n.d.	n.d.	n.d.	n.d.
CYP1A2	11143	11002	9572	17340
CYP2B	n.d.	20122	n.d.	26831
CYP3A	3391	14216	5758	19975

Units are relative area units derived from band intensities. n.d.: Not detected. These data were not analyzed for statistical significance. Data are group means.

The CARC concluded that in vivo data support an increase in microsomal protein content and PROD and BROD activity in rats for both metolachlor and S-metolachlor (Associative Events #1 and #2).

Associative Event #3: Increased liver weight.

a. 14-day mechanistic study in female rats (MRID 49927706) – *S-Metolachlor*

Liver weights from the 14-day study are presented in Table 9. Terminal body weights were decreased ($p<0.05$) at 5000 ppm by 11% on Day 4, by 8% on Day 8, and were similar to controls on Day 15. Absolute liver weights were decreased ($p<0.05$) by 11% on Day 2, but were increased ($p<0.05$) by 14% on Day 8 at 3000 ppm and by 17% on Day 15 at 5000 ppm. At ≥3000 ppm, adjusted liver weights were increased ($p<0.05$) by 10-25% on Days 3, 4, 8 and 15. Relative to body liver weights were similar to controls for all time points and dose groups. There were no effects of treatment on liver weights at 300 ppm at any time point.

Treatment with 1200 ppm PB decreased ($p<0.05$) terminal body weights by 10% on Day 4 and 8% on Day 8, increased ($p<0.01$) absolute liver weights by 21% on Day 8, and increased ($p<0.01$) adjusted liver weights by 23% on Day 4 and by 33% on Day 8.

TABLE 9. Absolute, adjusted, and relative (to body) liver weights in female rats treated with S-metolachlor in the diet for up to 15 days.[a]

Parameter	Dose (ppm)				
	0	300	3000	5000	Positive control[b]
Day 2					
Terminal body weight (g)	159.14±8.20	161.97±9.45	149.02±8.43* (↓6)	150.95±6.83	---
Absolute (g)	7.388±0.448	7.741±0.930	6.672±0.538	6.601±0.608* (↓11)	---
Adjusted (g)	**7.19±0.53**	**7.40±0.56**	**6.99±0.55**	**6.82±0.53**	---
Relative to body (%)	4.65±0.28	4.77±0.36	4.48±0.27	4.37±0.37	---
Day 3					
Terminal body weight (g)	172.96±8.59	173.96±8.73	173.07±8.26	165.32±7.55	---
Absolute (g)	7.970±0.870	7.853±0.785	8.805±1.083	8.487±1.081	---
Adjusted (g)	**7.84±0.72**	**7.64±0.73**	**8.67±0.72* (↑11)**	**8.96±0.77** (↑14)**	---
Relative to body (%)	4.60±0.35	4.51±0.41	5.08±0.46	5.12±0.52	---
Day 4					
Terminal body weight (g)	200.76±10.21	195.15±13.13	199.03±6.91	177.68±9.25** (↓11)	180.43±8.62** (↓10)
Absolute (g)	8.408±1.105	7.853±0.694	9.117±0.682	8.364±0.800	8.763±0.766
Adjusted (g)	**8.01±0.71**	**7.75±0.67**	**8.81±0.69* (↑10)**	**9.16±0.85* (↑14)**	**9.46±0.91** (↑23)[c]**
Relative to body (%)	4.18±0.43	4.03±0.28	4.58±0.26	4.70±0.32	4.85±0.29
Day 8					
Terminal body weight (g)	209.09±8.49	208.42±13.16	205.33±11.99	191.85±15.07* (↓8)	191.95±12.61** (↓8)
Absolute (g)	8.621±0.683	8.821±1.041	9.790±0.832* (↑14)	9.539±0.945	10.433±1.168** (↑21)
Adjusted (g)	**8.36±0.68**	**8.59±0.67**	**9.71±0.66** (↑16)**	**10.11±0.74** (↑21)**	**10.88±0.92** (↑33)[d]**
Relative to body (%)	4.12±0.31	4.23±0.33	4.77±0.29	4.97±0.34	5.43±0.44
Day 15					
Terminal body weight (g)	227.21±13.73	237.29±17.94	222.17±11.29	217.20±18.10	---
Absolute (g)	8.965±1.264	9.133±1.100	9.776±1.578	10.487±1.414* (↑17)	---
Adjusted (g)	**8.88±0.82**	**8.34±0.87**	**10.04±0.82** (↑13)**	**11.10±0.85** (↑25)**	---
Relative to body (%)	3.94±0.39	3.84±0.27	4.38±0.50	4.82±0.39	---

a Data were obtained from Tables 5.1 through 5.7 on pages 58-64 of MRID 49927706. Percent difference from controls (presented in parentheses) were calculated by the reviewers.
b Positive control = 1200 ppm phenobarbital (PB). n=20 for Days 1-4; n=10 for Day 8.
c Percent of control calculated against adjusted control value 7.71.
d Percent of control calculated against adjusted control value 8.17.
* Significantly different from controls; $p<0.05$.
** Significantly different from controls; $p<0.01$.
--- Not recorded (all rats euthanized by Day 8)

b. 28-day dietary study in rats (MRIDs 50396001 and 50396002) – *S-metolachlor and Metolachlor*

Groups of 5 rats/sex/dose (Tif: RAIf [SPF]) were administered metolachlor or S-metolachlor via the diet at concentrations of 0, 30, 300, 3000 and 5000 ppm for 28 days (Fankhauser, 1995, MRID 50396001; Persohn, 1995, MRID 50396002). An additional 3 rats/sex/dose were terminated after 7 days of dietary administration and an additional 5 rats/sex/dose (3000 and 5000 ppm only) were terminated after a 28-day recovery period. Only data for female rats are described below.

Treatment with either compound for 28 days at doses ≥3000 ppm resulted in increased absolute and/or relative liver weights and increased incidences of hepatocellular centrilobular hypertrophy in female rats. These data are summarized in Table 10.

Table 10

Liver findings in female rats following 28 days dietary administration of metolachlor or *S*-metolachlor. *: Statistically significantly different from control with p≤0.05.

Compound	Dose level (ppm)	Mean absolute liver weight (g)	Mean relative liver weight (% of bodyweight)	Hepatocellular centrilobular hypertrophy (n/5)
Metolachlor	0	8.331	4.091	0
	30	8.617	4.126	0
	300	9.068	4.163	0
	3000	9.262	4.507*	3
	5000	10.12*	4.709*	4
S-metolachlor	0	8.331	4.091	0
	30	8.343	4.067	0
	300	8.091	4.008	0
	3000	9.422*	4.601*	2
	5000	8.741	4.443	3

*: Statistically significantly different from control with p≤0.05.

c. 90-day dietary study in rats – *S-metolachlor and Metolachlor*

In a 3-month dietary toxicity study (Frankhauser, 1999, MRID 44775402), groups of male and female Sprague-Dawley rats (20/sex for controls, 10/sex/ treated group) were given CGA-77102 (S-metolachlor) (a.i. 98.5 %, Lot/Batch P.501001) administered in feed at 0, 30, 300 or 3000 ppm (equivalent to 0, 1.90, 20.4 and 208.0 mg/kg/day for males and 0, 2.13, 23.9 and 236.0 mg/kg/day for females).

In a 3-month dietary toxicity study (MRID 44775401), groups of male and female Sprague-Dawley rats (20/sex for controls, 10/sex/ treated group) were given CGA-24705 (metolachlor) (a.i. 97.7 %, Lot/Batch P.111072) administered in feed at 0, 30,300 or 3000 ppm (equivalent to 0, 2.00, 20.2 and 210 mg/kg/day for males and 0, 2.32, 23.4 and 259 mg/kg/day for females).

For S-metolachlor, liver/ body weight was statistically increased in 3000 ppm females (+7%) (Table 11).

For metolachlor, statistically significant changes in absolute and relative organ weights were limited to decreased liver weight in 30 and 300 ppm females (-11 % and-12%, respectively), increased liver/body weight in 3000 ppm females (+9) (Table 11).

TABLE 11. Selected mean ± SD absolute and relative liver weights in rats fed S-metolachlor and metolachlor for 13 weeks.				
	Treatment Group (ppm)			
Organ weight	**0**	**30**	**300**	**3000**
Females (S-Metolachlor)				
Liver (g) **% body**	10.26 ± 1.280 3.804 ± 0.1667	9.945 ± 0.879 3.756 ± 0.1845	9.954 ± 1.218 3.634 ± 0.1661	10.30 ± 0.879 4.089 ± 0.2865** (+7%)a
Females (Metolachlor)				
Liver (g) **% body**	11.00±1.316 3.832±0.303	9.737±0.827* (-11%) 3.679±0.175	9.631±0.895* (-12%) 3.721±0.325	10.48±1.027 4.165±0.235** (+9%)a

Data taken from DERs for MRIDs 44775401 and 44775402.

** Statistically significant versus control, $p \leq 0.01$.

* Statistically significant versus control, $p \leq 0.05$.

[a] Percent difference from control calculated by reviewer.

d. Chronic toxicity/carcinogenicity study in rats (MRID 00129377)– *Metolachlor*

No statistically significant increases were seen in liver weights at 52 or 104 weeks (Table 12).

TABLE 12: Mean absolute, relative to body, and relative to brain organ weights in female rats administered metolachlor in the diet for up to 52 and 104 weeks. [a]					
Organ /tissue		**Dose level (mg/kg)**			
		0	**30**	**300**	**3000**
52 Weeks					
Terminal body weight (g)		386	---	---	371 (↓4)
Liver	Absolute (g)	10.2	---	---	10.3
	Relative (to body; %)	2.637	---	---	2.808
	Relative (to brain; %)	496	---	---	483
104 Weeks					
Terminal body weight (g)		511.5	528.0	531.9	461.3
Liver	Absolute (g)	14.0	13.7	13.7	13.7
	Relative (to body; %)	2.826	2.670	2.591	3.078
	Relative (to brain; %)	676.9	653.8	655.0	675.1

Data were obtained from Tables 34-36 on pages 142-145 of MRID 00129377.

The CARC agreed that sufficient data are presented for S-metolachlor and metolachlor which are consistent with Associative Events 1,2 and 3 in the registrants proposed MOA.

Key Event #3: Formation of Hepatocellular Foci

Chronic toxicity/carcinogenicity study in rats - *Metolachlor*

In a chronic toxicity/carcinogenicity study, male and female rats (Crl:CD [Sprague Dawley]) were administered metolachlor via the diet at concentrations of 0, 30, 3000 and 3000 ppm for up to 104 weeks (Tisdel et al., 1983, MRID 00129377). A statistically significant, increase in eosinophilic cell foci were also noted for noted for female rats treated with 3000 ppm for 104 weeks. The total incidence of foci of cellular alteration in the liver, including eosinophilic, clear, and basophilic foci, was also increased ($p<0.05$) in females (46/60 treated vs. 15/60 control). Non-neoplastic microscopic findings (foci of cellular alteration) are presented in Table 13.

TABLE 13: Incidence of non-neoplastic microscopic findings in the liver of female rats administered metolachlor in the diet for up to 104 weeks. [a]				
Study Week / interval	**Dose level (mg/kg)**			
	0	**30**	**300**	**3000**
Females				
Foci of cellular alteration				
Eosinophilic	4/60	7/60	5/60	23/60*
Clear	4/60	6/60	9/60	12/60
Basophilic	7/60	5/60	10/60	11/60
Total incidence	15/60	18/60	24/60	46/60*

a Data were obtained from text table on page 20 of MRID 00129377

* Significantly different from control; $p<0.05$.

The CARC concluded that there is evidence of increased altered eosinophilic foci in female rats treated with metolachlor at 3000 ppm, supporting Key Event #3.

Key Event # 4: Formation of Liver Tumors

In a chronic toxicity/carcinogenicity study, male and female rats (Crl:CD [Sprague Dawley]) were administered metolachlor via the diet at concentrations of 0, 30, 3000 and 3000 ppm for up to 104 weeks (Tisdel et al., 1983, MRID 00129377). Treatment related liver tumors were seen in female rats at 3000 ppm only. See Tables 1 and 2 for tumor incidences and statistical analyses.

IV. IPCS/ILSI Framework for the Evaluation of the Human Health Relevance of a Hypothesized Mode of Action

The following information was obtained from the registrant's MOA proposal for liver tumors in female rats (MRID 49927701).

Has the Mode of Action Been Established in the Animal Model?

A weight of evidence analysis for the animal MOA with *S*-metolachlor is described in the following sections.

Dose concordance of key and associate events

Table 14 summarizes the dose concordance of the key and associate events (as defined in the hypothesized MOA [see Figure 1]) with the tumor outcome. This table considers concordance with the following doses.

• ≤300 ppm: no liver tumors in female rats.

• ≥3000 ppm: 3000 ppm is the lowest observed effect level for liver tumors in female rats.

Table 14. Summary of dose concordance of key and associate events.

The ability of *S*-metolachlor to activate CAR has been explicitly demonstrated *in vitro*; evidence of CAR activation *in vivo* is inferred from induction of CYP2B/3A.

Table 14. Dose Concordance of Associative Events and (Causal) Key Events in the Proposed MOA - Female Rat Liver Tumors

Dose (ppm)	CAR Activation[a] (Key Event #1)	Early Transient Increase in Hepatocellular Proliferation[a] (Key Event #2)	Induction of *Cyp2B* Gene Expression/ increased CYP Enzyme Activity (CYP2B/3A)[a] [increased PROD/ BROD activities] (Associative)	SER Proliferation (Associative)	Increased liver weight (Associative)	Increased Incidence of Hepatocellular Foci (Key Event #3)	Liver tumors (Key Event #4)
S-Metolachlor							
≤300	Yes *in vitro*	No	Yes -small transient	No	No	No	No
≥3000	Yes *In vitro*	Yes	Yes - marked	Yes	Yes	Yes	Yes
Metolachlor							
≤300	--	No	No	No	No	--	--
≥3000	--	No	Yes	Yes	Yes	--	--

[a]Confirmed *in vitro* (CAR transactivation) and *in vivo* (inferred from observed increases in CYP2B expression) with S-metolachlor

-- = not measured

Table 14 demonstrates that it is only at the tumorigenic doses levels (≥3000 ppm) that all of the key (and associate events) events are observed. Effects at the non-tumorigenic dose levels (≤300 ppm) are confined to a small, transient increase in CYP2B/3A, indicative of a low level of CAR activation of insufficient magnitude to stimulate an increase in hepatocellular proliferation or any of the other key or associate events.

Based on the evidence presented, the CARC concluded that there is strong concordance between key (and associative) events and the dose level that produces tumors.

Temporal concordance of key and associative events (Table 15)

The CARC concluded that the key and associative events occur in a logical, time-dependent manner consistent with the proposed CAR MOA.

• Activation of CAR *in vivo* is evident after 1 day of exposure (based on increases in CYP2B/3A and hepatocellular proliferation).

• Increased hepatocellular proliferation is evident at early time points (after 1-3 days of exposure), but not at later time points (≥7 days of exposure).

• Increased CYP2B/3A is evident after 1 day of exposure.

• Increases in SER proliferation/hepatocellular hypertrophy and liver weight are evident after 2 to 3 days of exposure.

• In the chronic toxicity and carcinogenicity study, increased hepatocellular foci (eosinophilic) were first noted at 104 weeks of treatment.

• In the chronic toxicity and carcinogenicity study, a liver tumor (carcinoma) was observed at week 90.

TABLE 15. Temporal Concordance of Associative Events and (Causal) Key Events in the Proposed MOA - Female Rat Liver Tumors

Time	CAR activation (Key Event #1)	Early Transient Increase in hepatocellular proliferation (Key Event #2)	Induction of CYP2B/ 3A [increased PROD/ BROD activities] (Associative)	SER Proliferation/ hepatocellular hypertrophy (Associative)	Increased liver weight (Associative)	Altered hepatic foci (Key Event #3)	Liver tumors (Key Event #4)
S-Metolachlor							
1 day	Yes[a] *in vitro*	Yes	Yes	--	--	--	--
1-3 days	--	Yes	Yes	--	Yes	--	--
4-8 days	--	No	Yes	Yes	Yes	--	--
14-15 days	--	No	Yes	Yes	Yes	--	--
28 days	--	No	Yes	Yes	Yes	--	--
60 days	--	No	--	--		--	--
90 days	--	--	--	Yes	Yes	--	--
1 year	--	--	--	--	--	--	--
1.5-2 years	--	--	--	--	--	--	--
Metolachlor							
1 day	--	--	--	--	--	--	--
1-3 days	--	No	--	--	--	--	--
4-8 days	--	No	--	--	--	--	--
14-15 days	--	No	Yes	--	--	--	--
28 days	--	No	Yes	Yes	Yes	--	--
60 days	--	No	Yes	--	--	--	--
90 days	--	--	--	Yes	Yes	--	--
1 year	--	--	--	--	No effect	--	--
1.5-2 years	--	--	--	--	No effect	Yes	Yes

[a]CAR transactivation confirmed *in vitro* and *in vivo* (inferred from observed increases in CYP2B expression) with S-metolachlor
"—" no data

Reproducibility and consistency

The CARC agreed with the registrant that the data described show high levels of both consistency and reproducibility of the key and associative events. Particular examples include:

• Consistent observation of increases in CYP2B/3A at tumorigenic dose levels.

• The lack of an effect on hepatocellular proliferation at >3 days of exposure was reproducible.

• When both metolachlor and S-metolachlor were tested, both compounds produced similar data.

There was a lack of an increase in hepatocellular proliferation noted in female rats administered 3000 ppm metolachlor for 3 days in the 60-day dietary administration mode of action study (Mainwaring, 2006, MRID 50396003). This is inconsistent with the data generated in the 14-day dietary administration mode of action study, where clear increases in hepatocellular proliferation were noted for animals receiving ≥3000 ppm *S*-metolachlor for 1-3 days (Dhinsa, 2014, MRID 49927706). Both of these studies used a BrdU incorporation technique, but differed in their approach. The 14-day study can be considered more reliable as the amount of BrdU the animals received was tightly controlled (a known amount relative to bodyweight was administered by subcutaneous injection 2 hours prior to sacrifice) and BrdU incorporation and staining were checked by ensuring adequate staining of a section of duodenum (a highly proliferative tissue) that was processed and evaluated concurrently with the liver. In addition, this study evaluated the effects of a positive control compound (sodium phenobarbital), which gave the expected response. In contrast, in the 60-day study, BrdU was administered to the animals by use of osmotic mini-pumps 3 days prior to sacrifice. Such a delivery system will result in a more variable exposure between animals as the rate of administration is the same for each animal and is not adjusted for bodyweight. Furthermore, when using such devices, it is critical that appropriate controls are in place to evaluate delivery, such as determination of the concentration of BrdU in the blood, the use of a positive control compound and qualitative evaluation of BrdU incorporation into a section of a highly proliferative tissue evaluated concurrently with the liver.

The results of the 14-day study are supported by the result of the four acute dosing studies, in which increases in hepatocellular proliferation were noted.

Biological plausibility

The liver is the most common target tissue affected in cancer bioassays (Gold *et al*., 2001). This may be due to the fact that the liver is the major site of metabolic activation of chemicals, and also the first organ exposed to any chemical following dietary administration and absorption from the gastrointestinal tract. The MOA for liver tumor formation in rodents by activation of CAR is well established (Whysner *et al*., 1996; Holsapple *et al*., 2006; Elcombe *et al*., 2014) and includes

increased liver weight, SER proliferation, hepatocellular hypertrophy (invariably in the centrilobular region) and a transient stimulation of hepatocellular proliferation.

The S-metolachlor/metolachlor-induced key and associative events summarized are similar to those seen with other CAR-activating compounds and, therefore, the CARC concluded that the proposed MOA for S-metolachlor/metolachlor-induced liver tumors is biologically plausible.

Alternative mode of action hypotheses

There are a number of previously described MOAs for liver tumors in rodents established in the literature. However, when the toxicity database for *S*-metolachlor was assessed for the key events associated with each potential MOA, none were found to be supported by the data. A summary of these alternative MOAs proposed by the registrant and the relevant data for *S*-metolachlor are provided in Table 16.

Table 16. Alternative modes of action for induction of liver tumors in rodents and reason(s) for their exclusion.

Alternative MOA	Reason for Exclusion
Genotoxicity	*S*-metolachlor has been tested in a wide variety of *in vitro* and *in vivo* assays for genotoxicity. There is no evidence that S-metolachlor is genotoxic. See Table 17.
Peroxisome proliferator	Treatment with S-metolachlor did not increase female rat hepatic peroxisomal fatty acid β-oxidation, lauric acid 11-/12-hydroxylation activity or CYP4A protein expression (Persohn, 1995, MRID 50396002; Mainwaring, 2006, MRID 50396003).
Enzyme induction (aryl hydrocarbon receptor [AhR]-mediated)	Treatment with *S*-metolachlor did not result in increased EROD or MROD activities or CYP1A isoform expression of magnitudes suggesting activation of the AhR (Persohn, 1995, MRID 50396002); Mainwaring, 2006, MRID 50396003).
Estrogenic stimulation	In the large mammalian toxicological database available for metolachlor/*S*-metolachlor, including the studies summarized in this document, as well as studies of the effects of S-metolachlor on reproduction and development (Lightkep, 1980, MRID 00041283; Smith *et al*., 1981, MRID 00088091; Lochry, 1985, MRID 00151941; Gilles and Giknis, 1995, MRID 43928924; Khalil, 1995, MRID 43928925), there is no evidence for estrogenic stimulation. There was no convincing evidence of potential interaction of metolachlor with the estrogen pathway. Metolachlor was negative in the *in vitro* estrogen receptor (ER) binding and the ER transactivation assay (ERTA), gave an equivocal response in the aromatase assay, but potential issue with protein (receptor) denaturation and/or compound solubility) and induced a marginal increase (1.2- to 1.3-fold) in estradiol production in the steroidogenesis assay. No treatment-related estrogenic effects were seen in the *in vivo* uterotrophic or female pubertal assays [EPA-HQ-OPP-2014-0772-0022].
Statins	*S*-metolachlor was not designed to inhibit HMG-CoA reductase, nor is there any evidence to suggest that cholesterol levels were found to be increased in rats following *S*-metolachlor administration.
Cytotoxicity	Following administration to female rats, *S*-metolachlor did not produce elevations in markers of hepatocyte damage nor was there any evidence of cytotoxicity or regenerative proliferation (the proliferation noted following treatment with *S*-metolachlor was transient and not sustained).
Infection	Following administration to female rats, *S*-metolachlor did not produce any signs of hepatic infection, cytotoxicity or regenerative proliferation. The proliferation noted following treatment with *S*-metolachlor was transient and not sustained.
Iron/copper overload	Following administration to female rats, *S*-metolachlor did not produce elevations in markers of hepatocyte damage nor was there any evidence of cytotoxicity or regenerative proliferation.
Increased apoptosis	There was no consistent evidence that administration of metolachlor increased female rat hepatic apoptosis (Mainwaring, 2006, MRID 50396003).

Formation of dialkylbenzoquinone imine	Metolachlor is unlike other chloroacetamide herbicides (acetochlor and alachlor), in that it is not metabolized to form a dialkylaniline metabolite or the DNA-reactive dialkylbenzoquinone imine. (Dapson and Rinde, 1994, TXR No. 0011347). Consistent with this difference, *S*- metolachlor only produced one tumor type (liver tumors in female rats) and showed no evidence of genotoxicity.

Note: This table was excerpted/adapted from the registrant's weight of evidence document, MRID 49927701.

Evaluation of Potential Genotoxicity

In its prior review of the *in vitro* and *in vivo* genotoxicity studies, including additional *in vivo*/*in vitro* Unscheduled DNA Synthesis (UDS) and a cell proliferation assays per request of the EPA carcinogenicity peer review committee (EPA 1994), the CPRC concluded that racemic metolachlor and *S*-metolachlor were negative in the mutagenicity studies (Dapson, S and Rinde E, 11/16/1994, TXR No. 0011347). A summary of these genotoxicity studies and their findings for metolachlor and *S*-metolachlor are provided in Table 17.

Table 17. Summary of Genotoxicity Studies with Racemic Metolachlor and *S*-Metolachlor.

Study Type	Test System	Test chemical Concentration or	Result	Ref.
Gene mutation (Ames Test)	*Salmonella typhimurium*; Strains TA 98, TA 100, TA 102, TA 1535, TA	**S-Metolachlor** 312.5 - 5000 µg/plate (original experiment) 78.13 - 5000 µg/plate (confirmatory	Negative	Hertner, 1995b MRID 43928927
Cytogenetics Micronucleus assay	Mouse	**S-Metolachlor** 500, 1000 or 2000 mg/kg	Negative	Hertner, 1995a MRID 43928926
Unscheduled DNA Synthesis	Rat	**S-Metolachlor** 500, 1500, 3200 (females), 5000 (males) mg/kg	Negative	Hertner, 1995c MRID 43928928
In vitro Gene mutation (Ames Test)	*Salmonella typhimurium*; Strains TA 98, TA 100, TA 1535, TA 1537	**Metolachlor** 10, 100, 1000, 10000 µg/plate	Negative	Arni, 1976 MRID 00015397
Gene mutation	Mouse lymphoma L5178Y/TK	**Metolachlor** without activation: 9.5 - 190 nl/ml; with activation; 10.5 to 280 nl/ml	Negative	Beilstein, 1984 MRID
Cytogenetics micronucleus assay	Chinese Hamster	**Metolachlor** 0, 1250, 2500 or 5000 mg/kg	Negative	Strasser, 1986 MRID 00158925
***In vivo* in germ cells** Dominant Lethal assay	Mouse	**Metolachlor** 100 and 300 mg/kg (single application)	Negative	Fritz, 1976 MRID 00015630

Study Type	Test System	Test chemical Concentration or	Result	Ref.
DNA Damage/Repair	Rat Hepatocytes	**Metolachlor** 0.25, 1.25, 6.25, 31.25 nl/ml	Negative	Puri, 1984b MRID 00142828
DNA Damage/Repair	Human fibroblasts	**Metolachlor** 0.125, 0.625. 3.125 or 15.625 nL/mL	Negative	Puri, 1984a MRID 00142827
UDS assay	Rat hepatocytes	**Metolachlor** 500-1500 (f), 1250-4000 mg/kg (m)	Negative	Ham, 1994 MRID

Note: The registrant cited additional genotoxicity studies that were negative. These studies were not included here since they were not formally submitted or reviewed by the Agency.

Formation of dialkylbenzoquinone imine (as proposed by the registrant)

Two members of the chloroacetamide class of herbicides are tumorigenic to mice and rats via formation of the DNA-reactive metabolite dialkylbenzoquinone imine. Alachlor is classified by the US EPA as a "likely human carcinogen" based on increased incidence of stomach, thyroid and nasal tumors in rats and lung tumors in male and female mice (US EPA, 1998). Acetochlor is classified by the US EPA as "suggestive evidence of carcinogenic potential" based on weak evidence for benign lung tumors in male and female mice and histiocytic sarcomas in female mice and acceptable mode of action data for the rat tumors (nasal olfactory epithelial tumors and thyroid follicular cell tumors) (US EPA, 2007). In contrast, metolachlor currently was classified by the US EPA as Group C, possible human carcinogen, based on increased liver adenomas and combined adenomas/carcinomas in female rats. As noted in a review by the EPA (1994), the tumorigenic MOA for alachlor and acetochlor involves extensive amide dealkylation and/or deacylation, formation of a disubstituted aniline, and ultimate metabolism to the DNA-reactive dialkylbenzoquinone imine. The EPA has also concluded that metolachlor does not share this MOA, based on extensive metabolism and mechanistic studies with metolachlor that were submitted by the Registrant. The branched N-alkyl group of metolachlor creates steric hindrance, decreasing susceptibility to amide dealkylation and hydrolysis, greatly limiting (or completely preventing) the formation of the disubstituted aniline metabolite.

Uncertainties, inconsistencies and data gaps

The available data support the proposed MOA for the increased incidence of rat liver tumors in female rats as shown in Figure 1 and exclude the alternative MOAs described in Table 15. In the absence of a long-term carcinogenicity study with S-metolachlor, the tumorigenic effects of metolachlor can be reasonably explained by CAR activity demonstrated in the MOA for S-metolachlor. This idea is supported by the comparable effects of S-metolachlor and metolachlor on CYP2B expression/BROD activity and liver hypertrophy.

Are the Key Events in the Animal Mode of Action Plausible in Humans?

The registrant also provided information to support their conclusions that the CAR-mediated MOA for liver tumors in rodents is not plausible in humans. Following establishment of a plausible MOA for the induction of liver tumors in female rats, the next step is to assess the relevance to humans by assessing the qualitative and, if necessary, quantitative differences between the rat and human for the associative and causal key events as described in the IPCS/ILSI framework (Sonich-Mullin *et al*., 2001; Meek *et al*., 2003, 2014; Boobis *et al*., 2006).

Qualitative differences in key events

S-metolachlor was shown to be a potent direct activator of rat CAR and a weaker activator of human CAR *in vitro*. Therefore, it can be concluded that the human is not qualitatively different to the rat with respect to the causal key event of CAR activation following treatment with S-metolachlor, although a clear quantitative difference was observed.

To explore the species differences in response to S-metolachlor, an *in vitro* investigative study using primary hepatocytes isolated from female Sprague Dawley rats was conducted to assess the effects of S-metolachlor on PROD and BROD (CYP2B/3A) activities and hepatocellular proliferation (Elcombe, 2014, MRID 49927704), and a similar experiment was conducted with isolated female human hepatocytes (Elcombe, 2014, MRID 49927702).

In these experiments, hepatocytes were exposed to *S*-metolachlor at up to 75 µM for 96 h. 75 µM was selected as the highest concentration based on data from preliminary range-finding studies, in which concentrations >75 µM resulted in excessive cytotoxicity (Elcombe, 2014c, MRID 49927705; Elcombe, 2014, MRID 49927703). Testing up to the highest tolerable concentration allows for a robust assessment of the intrinsic potential of *S*-metolachlor to induce CYP2B/3A activity and hepatocellular proliferation. These experiments also included appropriate controls: sodium phenobarbital as a known inducer of PROD/BROD activities in both species and an inducer of proliferation in rat and epidermal growth factor (EGF) as a known inducer of proliferation in both species.

Table 17 provides a summary of the data from the two studies and shows that treatment with *S*-metolachlor caused induction of CYP2B/3A activity (observed as an increase in BROD activity) and a proliferative response in the rat hepatocytes, but not the human hepatocytes.

Both control compounds gave the expected responses for both species, indicating the test systems were responding as expected.

Table 17. Qualitative comparison of the effects of *S*-metolachlor on selected parameters in female Sprague Dawley rat and human hepatocytes *in vitro.*

	***S*-metolachlor**	
	Rat	**Human**
CYP2B/3A activity	↑	---
Hepatocellular proliferation	↑	---

Key: ↑ = Parameter significantly increased by treatment,
--- = Parameter unaffected by treatment.

The registrant stated that the summary data for S-metolachlor, indicate that the human is qualitatively different from the rat with respect to the key event of increased hepatocellular proliferation following treatment with *S*-metolachlor.

The registrant concluded that the human relevance of the MOA can be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans for S-metolachlor.

However, the Agency determined that the human CAR receptor is activated by S-metolachlor as seen in *in vitro* studies and, therefore, can be relevant to humans qualitatively. Furthermore, there is limited data to determine if *in vivo* human hepatocyte models would continue to express receptors in culture and the statement does not take into consideration diversity that occurs across human population subgroups. Consistent with current HED policy, the CARC considers the CAR MOA to be plausible in humans.

V. Committee's Assessment of the Weight-Of-The-Evidence for the CAR MOA for Liver Tumors

On July 19, 2017, the CARC reconvened to evaluate the mechanistic data on S-metolachlor to support a proposed MOA for liver tumors in female rats. From these deliberations, the CARC drew the following conclusions for the rat liver tumor MOA:

The proposed key events for this MOA are:

1. Activation of the constitutive androstane receptor (CAR). **(Key Event #1)**
2. An early, transient, increase in hepatocellular proliferation. **(Key Event #2)**
3. Increased hepatocellular foci as a result of clonal expansion of spontaneously mutated

(initiated) cells. **(Key Event #3)**

4. Eventual progression to form liver tumors. **(Key Event #4)**

The CARC made the following conclusions based on the available data:

- There is evidence that S-metolachlor causes direct activation of CAR (mouse, rat, and human) nuclear receptors as evidenced by *in vitro* gene expression to support Key Event #1. There is *in vivo* data supporting an increase in microsomal protein content (rat), and PROD and BROD activity (rat) for both metolachlor and S-metolachlor.

- There is evidence of S-metolachlor induced hepatocellular proliferation in rats. In addition, there is a robust initial proliferative burst in the rat BrdU labelling experiment and strong evidence for CAR induction as noted above. These data adequately support Key Event #2.

- There is evidence of increased altered eosinophilic foci in the rat with metolachlor, supporting Key Event #3.

- The data also support the associative events of increased liver weight and hepatocellular hypertrophy.

- There is appropriate concordance between the dose causing tumors (3000 ppm female rats) and the dose response and temporal associations for the key events and associative events.

- Alternative MOAs (*i.e.,* genotoxicity, cytotoxicity, peroxisome proliferation, estrogenic stimulation, statins, infections, iron/copper overload, increased apoptosis, formation of dialkylbenzoquinone imine, and mitogenesis induced by other nuclear receptors such as AhR or PPAR α) have been adequately ruled out.

Overall, the CARC concluded that the weight of evidence supports a CAR-mediated mitogenic MOA for metolachlor/S-metolachlor-related liver tumors in the female rat.

VI. Epidemiology Evidence from the Agricultural Health Study

Epidemiology evidence from the Agricultural Health Study (AHS) regarding exposure to

metolachlor and risk of liver cancer was evaluated (Woods, S., D444122, 10/16/2017). The Agricultural Health Study (AHS) is a federally funded study that evaluates associations between pesticide exposures and cancer and other health outcomes and represents a collaborative effort between the US National Cancer Institute (NCI), the National Institute of Environmental Health Sciences (NIEHS), Centers for Disease Control and Prevention (CDC's) National Institute of Occupational Safety and Health (NIOSH), and the US EPA. The AHS participant cohort includes more than 89,000 licensed commercial and private pesticide applicators and their spouses from Iowa and North Carolina. Enrollment occurred from 1993 – 1997, and data collection is ongoing. The AHS maintains a list of publications resulting from AHS studies[1]. The epidemiology review found that the evidence gathered from AHS studies that reported on the association between metolachlor exposure and risk of liver cancer was inadequate to conclude the presence or absence of a causal relationship. At this time, only a single AHS study presented estimates of effect for metolachlor on liver cancer risk (Silver *et al.* (2015)). The strength of the evidence from Silver *et al.* (2015) is constrained by small numbers of exposed cases and other study limitations. The Agency will continue to monitor the epidemiology data arising from the AHS and other sources, and if a concern is triggered, additional analysis will be conducted.

VII. Classification of Carcinogenic Potential

In accordance with the EPA's Final Guidelines for Carcinogen Risk Assessment (March, 2005), the CARC concludes that metolachlor/S-metolachlor should be re-classified as "Not Likely to be Carcinogenic to Humans" at doses that do not induce cellular proliferation in the liver. This classification was based on convincing evidence that a non-genotoxic CAR-mediated mitogenic mode of action has been established for liver tumors in female rats. Additionally, there were no treatment related tumors observed in male rats or male or female mice and there is no concern for mutagenicity.

VIII. Quantification of Carcinogenic Potential

Based on this cancer classification, the quantification of cancer risk using a Q_1* approach is not required. A non-linear approach (*i.e.,* RfD) would adequately account for all chronic toxicity, including carcinogenicity, that could result from exposure to metolachlor/S-metolachlor. Accordingly, the RfD should be protective of the dose which induced hepatocellular proliferation in the female rat (150 mg/kg/day or 3000 ppm).

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